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2. Number of the Inventions stated in Extent of Claim
for Patent: 4

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6. List of annexed Documents:

(1) Specification	one set
(2) Drawings	one set
(3) Power of Attorney	one set
(4) Copy of this Application for Patent	one set

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SPECIFICATION

1. Title of the Invention

Novel DNA

2. Extent of Claim for Patent

- (1) A DNA which contains the polynucleotide of the nucleotide sequence 831-1485 as shown in Figure 1.
- (2) A DNA according to Claim 1, wherein the polynucleotide of the nucleotide sequence 490-830 as shown in Figure 1 or a fragment thereof is linked to the 5' end of the polynucleotide of the nucleotide sequence 831-1485 as shown in the same Figure.
- (3) A DNA according to Claim 2, wherein the polynucleotide of the nucleotide sequence 88-489 as shown in Figure 1 or a fragment thereof is linked to the 5' end.
- (4) A DNA according to Claim 3, wherein the polynucleotide of the nucleotide sequence 1-87 as shown in Figure 1 or a fragment thereof is linked to the 5' end.
- (5) A DNA according to any of Claims 1 to 4, wherein it has ATG at the 5' end without any reading frame shift.
- (6) A DNA according to Claim 1, which codes for the polypeptide of the amino acid sequence 278-494 as shown in Figure 2.
- (7) A DNA according to Claim 2, which codes for a polypeptide wherein the polypeptide of the amino acid sequence 164-277 as shown in Figure 2 or a fragment thereof is linked to the N terminus of the polypeptide of the amino acid sequence 278-494 as shown in the same Figure.
- (8) A DNA according to Claim 3, which codes for a polypeptide wherein the polypeptide of the amino acid sequence 30-163 as shown in Figure 2 or a fragment thereof is linked to the N terminus of the polypeptide as defined in Claim 7.
- (9) A DNA according to Claim 4, which codes for a polypeptide wherein the polypeptide of the amino acid sequence 1-29 as shown in Figure 2 or a fragment thereof is linked

to the N terminus of the polypeptide as defined in Claim 8.

(10) A DNA according to any of Claims 1 to 9, which codes for a polypeptide having Met at the N terminus thereof.

(11) A DNA according to any of Claims 1 to 10, which codes for a polypeptide equivalent in immunological or biological activities to the human immunoglobulin E H Chain.

(12) A DNA according to any of Claims 1 to 11, which forms part of a recombinant DNA molecule.

(13) A DNA according to any of Claims 1 to 12, which is linked downstream from a promoter.

(14) A DNA according to Claim 13, wherein the promoter is tryptophane promoter.

(15) A method of producing a DNA containing a polynucleotide of the nucleotide sequence 831-1485 as shown in Figure 1, which comprises reversely transcribing a mRNA coding for the human immunoglobulin E H Chain.

(16) A transformant which contains a DNA containing a polynucleotide of the nucleotide sequence 831-1485 as shown in Figure 1.

(17) A transformant according to Claim 16, which is Escherichia coli.

(18) A method of producing a polypeptide of or equivalent in immunological or biological activities to the human immunoglobulin E H chain, which comprises growing a transformant which contains a DNA containing a polynucleotide of the nucleotide sequence 831-1485 as shown in Figure 1, accumulating the polypeptide of or equivalent in immunological or biological activities to the human immunoglobulin E H chain and recovering the same.

3. Detailed Description of the Invention

This invention relates to a novel DNA. More particularly, this invention relates to a DNA containing a polynucleotide which codes for the human immunoglobulin E H-chain polypeptide, to a transformant carrying said DNA, and to a method for producing the human immunoglobulin E H-chain polypeptide by the cultivation of said transformant.

Immunoglobulins, which are present in animal body fluids and are closely associated with antibodies, consist of H (heavy) chains and L (light) chains. Each chain comprises the V region, which is determinative of the binding specificity with antigen, and the C region, which is determinative of the effector function. On the basis of the constituents of the H chains, immunoglobulins (Ig) are classified into 5 classes, namely A, D, G, M and E.

Among them, immunoglobulin E (hereinafter referred to as IgE), which constitutes reagin, has a molecular weight of 196,000 daltons and consists of two 75,000-dalton H chains and two 22,500-dalton L chains (in the case of human IgE), the chains being linked together by disulfide bond. The C region of the H chain of IgE comprises four sites, CH1 to CH4, and two H chains are linked together at CH2 by disulfide bonds. IgE is in charge of important biological reactions, such as allergic reactions. For instance, it is known that allergic reactions are induced by binding of specific antigen-bound IgE to sensitized mast cells or basophilic cells [K. Ishizaka and T. Ishizaka, Immunological Rev., 41, 109 (1978)]. Therefore, for the purpose of suppressing allergic reactions, the use of an IgE molecule having no antigen-binding site has been proposed. However, many problems remain unsolved with respect to a variety of in vivo reactions induced by IgE. One reason is that a sufficient quantity of human IgE cannot be supplied.

On the other hand, the anti-IgE antibody is an essential material in the diagnosis of allergic diseases and is demanded in very large quantity. For its production, however, the purified human IgE is required in large quantity. For this and other reasons, development of a technique capable of producing the human IgE on large scale and at low cost has been waited for.

In a so-far proposed method of producing IgE, the supernatant of a culture of human IgE-producing myeloma cells of an established line is treated for the separation of IgE followed by purification. However, said method involves cell culture and the cell growth rate is low. For these and other reasons, it is difficult to obtain a large quantity of IgE at low cost.

The present inventors have already succeeded in isolating a human IgE-encoding mRNA from cells (Japanese Patent Application No. 120,555/1981 filed July 30, 1981).

With the above mRNA, the present inventors continued their research with use of the technology of gene manipulation so that they could develop a technology of producing the human IgE H chain polypeptide by cloning the gene coding for the human IgE H chain polypeptide and introducing the thus-obtained recombinant DNA molecule into a host organism, and, as a result, they have completed the present invention.

Thus, the present invention provides a DNA which contains a polynucleotide coding for the human IgE H-chain polypeptide, a transformant carrying said DNA, and a method of producing the human IgE H-chain polypeptide or a polypeptide equivalent thereto in immunological or biological activities, which comprises growing the transformant carrying said DNA.

The DNA provided by the present invention is a DNA containing a polynucleotide having the nucleotide sequence shown in Figure 1.

Referring to Fig. 1, the polynucleotide of the

nucleotide sequence 831-1485 codes for a polypeptide of the amino acid sequence 278-494 as shown in Fig. 2. Thus, it codes for CH3-CH4 of the human IgE H-chain.

The polynucleotide of the nucleotide sequence 490-1485 as shown in Fig. 1 codes for a polypeptide of the amino acid sequence 164-494 as shown in Fig. 2, hence CH2-CH4 of the human IgE H-chain.

The polynucleotide of the nucleotide sequence 88-1485 as shown in Fig. 1 codes for a polypeptide of the amino acid sequence 30-494 as shown in Fig. 2. Said polypeptide covers the CH1-CH4 polypeptides of the human IgE H-chain.

Similarly, the polynucleotide of the nucleotide sequence 1-1485 as shown in Fig. 1 codes for a polypeptide of the amino acid sequence 1-494 as shown in Fig. 2. Said polypeptide includes the human IgE H-chain CH1-CH4 polypeptides.

For the direct expression, the above-mentioned polynucleotides may possess the codon ATG at the 5'-end thereof without reading frame shift. In that case, said polynucleotides code for polypeptides possessing Met at the N-terminus thereof.

The above-mentioned polynucleotides, with or without ATG at the 5'-end thereof without reading frame shift, are preferably linked at a site downstream from a promoter. The promoter includes, among others, the tryptophan synthesis (trp) promoter, rec A promoter and lactose promoter. Among these, the trp promoter is preferable.

Table 1 gives the definition of each symbol as used in the present specification, drawing and claims.

Table 1

DNA	= deoxyribonucleic acid
cDNA	= complementary deoxyribonucleic acid
RNA	= ribonucleic acid
mRNA	= messenger ribonucleic acid

A = deoxyadenylate
T = thymidylate
G = deoxyguanylate
C = deoxycytidylate
U = uridylate
dATP = deoxyadenosine triphosphate
dTTP = thymidine triphosphate
dGTP = deoxyguanosine triphosphate
dCTP = deoxycytidine triphosphate
ATP = adenosine triphosphate
EDTA = ethylenediamine tetraacetate
SDS = sodium dodecyl sulfate
Gly = glycine
Ala = alanine
Val = valine
Leu = leucine
Ile = isoleucine
Ser = serine
Thr = threonine
Cys = cysteine
Met = methionine
Glu = glutamic acid
Asp = aspartic acid
Lys = lysine
Arg = arginine
His = histidine
Phe = phenylalanine
Tyr = tyrosine
Trp = tryptophan
Pro = proline
Asn = asparagine
Gln = glutamine
bp = base pair(s)

In the present invention, a double-stranded DNA coding for the human IgE H-chain polypeptide can be produced by synthesizing a single-stranded cDNA using the mRNA coding for the human IgE H-chain polypeptide as produced by the method disclosed in Japanese Patent Application No. 120,555/1981 or a modification thereof as the template together with reverse transcriptase, for instance, then converting the cDNA to the double-stranded form, digesting the double-stranded DNA with an enzyme (exonuclease, endonuclease), adding an adapter to the digestion product, inserting the resulting product into a plasmid, introducing the plasmid into Escherichia coli, for instance, growing the thus-obtained transformant and isolating the cDNA-containing plasmid.

The mRNA to be used in the above process can be produced, for example, in the following manner.

Human myeloma cells of the established cell line U266, which are capable of producing human IgE, are cultivated, the proliferated cells are harvested by centrifugation, washed, for instance with physiological saline, and lysed in a denaturing solution, for instance N-laurylsarcosine buffer, with heparin, diethyl pyrocarbonate, etc. added, and an RNA fraction is collected in the conventional manner by, for example, layering the lysate onto 5.7 M CsCl solution followed by centrifugation and extraction with phenol. Then, polyadenylic acid-containing RNAs are separated using oligo(dT)-cellulose, poly(U)-Sephadex or the like. The subsequent sucrose density gradient centrifugation gives the mRNA.

Using the thus-obtained mRNA as the template, a single-stranded cDNA is synthesized by any method known per se with the use of reverse transcriptase, and the cDNA is further converted to the double-stranded form [Maniatis, T. et al., Cell, 8, 163 (1976)].

The double-stranded DNA is inserted into pBR 322 at the PstI or SphI restriction endonuclease cleavage site by, for example, the dG-dC or dA-dT homopolymer tailing method [Nelson, T. S., Methods in Enzymology, 68, 41

(1979), Academic Press Inc., New York]. Escherichia coli strain χ 1776, for instance, is transformed with the resulting recombinant plasmid. An adequate transformant can be selected on the basis of the tetracycline or ampicillin resistance.

The structural gene fragment for the human IgE H-chain has already been cloned [Nishida et al., Proc. Natl. Acad. Sci. USA, 79, 3833 (1982)], and its base sequence has been partially analyzed. This gene fragment (gift from Prof. Tasuku Honjo of Osaka University, Faculty of Medicine) is labelled with ^{32}P by, for example, the nick translation method [Rigby, P. W. J. et al., J. Mol. Biol., 113, 237 (1977)] or, alternatively, an oligonucleotide having the nucleotide sequence supposedly corresponding to the amino acid sequence of the human IgE H-chain polypeptide is synthesized chemically and labelled with ^{32}P . With the labelled product as the probe, the desired clone is secondarily screened out from among the already obtained tetracycline- or ampicillin-resistant transformants by the per se known colony hybridization method [Grunstein, M. and Hogness, D. S., Proc. Natl. Acad. Sci. USA, 72, 3961 (1975)]. The nucleotide sequence of the clone which gives a positive result in the above colony hybridization is determined by, for example, the method of Maxam-Gilbert [Maxam, A. M. & Gilbert, W., Proc. Natl. Acad. Sci. USA, 74, 560 (1977)] or the dideoxynucleotide synthetic chain termination method using phage M13 [Messing, J. et al., Nucleic Acids Res., 9, 309 (1981)], whereby the presence of the gene coding for the human IgE H-chain polypeptide can be confirmed. Then, the human IgE H-chain polypeptide-encoding gene can be cut out wholly or partly from the clone obtained and can be linked at a site downstream from an adequate promoter, the SD (Shine and Dalgarno) sequence and the translation start codon ATG, for introduction into an adequate host organism. The gene or part thereof can also be inserted into within

an adequate structural gene (e.g. β -lactamase gene or anthranilate synthetase gene) as inserted in a plasmid. In that case, the expression product is a chimera polypeptide coupled with the whole or part of the structural gene product.

The promoter includes those mentioned hereinabove, and the host organism includes bacteria such as Escherichia coli and Bacillus subtilis, among which Escherichia coli (e.g. strain 294, strain W3110), particularly strain 294, is preferred.

The strain 294 is a known strain [Backman, K. et al., Proc. Natl. Acad. Sci. USA, 73, 4174 (1976)] and has been deposited with the Institute for Fermentation, Osaka under deposit No. IFO-14171.

The transformation of a host organism with the DNA in the present invention is performed, for example, by the known method [Cohen, S. N. et al., Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)].

The thus-obtained transformant is cultivated in a per se known medium.

The medium is, for example, glucose- and Casamino acids-containing M9 medium [Miller, J., Experiments in Molecular Genetics, 431-433 (Cold Spring Harbor Laboratory, New York, 1972)]. An agent such as 3 β -indolylacrylic acid may be added as necessary for increased promoter efficiency.

The cultivation is generally conducted at 15-43°C for 3 to 24 hours. Aeration and/or stirring may be made as necessary.

After cultivation, cells are harvested by the known method and, for instance after suspending in a buffer, destructed by, for example, treatment with lysozyme or a surface active agent or ultrasonic treatment, followed by centrifugation to give a supernatant.

The human IgE H-chain polypeptide can be isolated from said supernatant by any of the generally known methods of purifying proteins, more advantageously by anti-human

IgE antibody column chromatography.

The human IgE H-chain polypeptide or a polypeptide equivalent thereto in immunological or biological activities as produced in the present invention is equivalent in immunological or biological activities to the human IgE H-chain polypeptide produced by the conventional method and can be used for the same purpose and in the same manner as the case where the conventional product is used.

Reference Example Isolation of human IgE-encoding mRNA
(1) Cultivation of U-266 cells

Human myeloma cells of the established cell line U-266 [Immunology, 38, 63 (1979)] (2.5×10^5 cells/ml) were cultivated in 500 ml of RPMI-1640 (Roswell Park Memorial Institute) medium with 10% fetal calf serum and 0.1 mg/ml each of penicillin and streptomycin (Takeda Chemical Industries) in a roller bottle at 37°C for 3 days.

(2) Preparation of polyadenylic acid-containing RNA

The total RNA extraction from U-266 cells was performed mainly by the method of Glisinet al. [Biochemistry, 13, 2633 (1974)]. Thus, U-266 cells after 3 days of were collected by centrifugation at 2,500 revolutions per minute for 5 minutes using a Sorvall centrifugal rotor GSA, suspended in physiological saline and again centrifuged at 2,500 revolutions per minute for 5 minutes for effecting cell washing. Five to ten volumes of 4% N-laurylsarcosine buffer (Wako Pure Chemical Industries) [2 mg/ml heparin (Wako Pure Chemical Industries), 0.2% diethyl pyrocarbonate (Tokyo Kasei), 0.01 M Tris·HCl, pH 7.6] was added to the cells, and the cells were mashed 15-20 times using a 30-ml Teflon homogenizer. To the resulting solution was added CsCl to a concentration of 0.5 g/ml, and the solution was layered on 7 ml of 5.7 M CsCl in a centrifugal tube for use in a Spinco SW27 rotor and centrifuged at 26,000 revolutions per minute for 20 hours for RNA sedimentation. The supernatant in the tube was sucked off, the upper part of the tube was cut off so as to leave the lower part thereof

(about 2 cm long), and the RNA sediment was dissolved in 0.4% N-lauroylsarcosine buffer. NaCl was added to the solution to a concentration of 0.2 M, and RNAs were precipitated at -20°C by adding cold ethanol to a final concentration of 70%.

(3) Fractionation by oligo(dT)-cellulose column chromatography

The ethanol-precipitated RNAs were collected by centrifugation on a Spinco SW27.1 rotor at 20,000 revolutions per minute for 20 minutes, and then dissolved in 10 ml of 10 mM Tris-HCl (pH 7.6)-0.5 M NaCl-1 mM EDTA-0.5% SDS buffer. A 10 cc syringe was packed with 4 ml (4 cm high) of oligo(dT)-cellulose dissolved in the same buffer. The above RNA solution was passed through this column and the eluent was again passed through the column to adsorb polyadenylic acid-containing RNAs. The column was washed with the same buffer until the ultraviolet absorption at 260 nm was no more detected, whereby unadsorbed RNAs were washed away. The polyadenylic acid-containing RNAs were then eluted from the column with 10 mM Tris-HCl (pH 7.6)-1 mM EDTA-0.3% SDS buffer (1 ml/fraction) while following the RNAs based on the absorption at 260 nm (O.D.). The RNA fractions were pooled and subjected to ethanol precipitation at -20°C .

(4) Fractionation by sucrose gradient centrifugation.

About 2 mg of the polyadenylic acid-containing RNAs obtained by the above procedure was layered on 10-30% sucrose density gradient solution in 0.05 M NaCl-0.01 M EDTA-0.01 M Tris-HCl (pH 7.6)-0.2% SDS buffer, and centrifuged at 24,000 revolutions per minute and at 20°C for 22 hours using an SW27 rotor. Thereafter, the contents were divided into 40 fractions and, for each fraction, the absorption at 260 nm (O.D.) were measured. The fractions were pooled by fives with an about 18S fraction at the center and subjected to ethanol precipitation. In this manner, the desired mRNA was obtained.

Example 1

(i) Synthesis of single-stranded DNA

A mixture of 5 µg of the mRNA as obtained in the above Reference Example, 100 units of reverse transcriptase (Life Science) and 100 µl of reaction mixture [5 µg of oligo(dT), 1 mM each of dATP, dCTP, dGTP and dTTP, 8 mM MgCl₂, 50 mM KCl, 10 mM dithiothreitol, 50 mM Tris·HCl, pH 8.3] was incubated at 42°C for an hour, then deproteinized with phenol, and treated with 0.1 N NaOH at 70°C for 20 minutes for decomposing and removing the RNA.

(ii) Synthesis of double-stranded DNA

The thus-synthesized single-stranded complementary DNA was maintained in 50 µl of a reaction mixture [the same reaction mixture as above except for the absence of the mRNA and oligo(dT)] at 42°C for 2 hours, whereby a double-stranded DNA was synthesized.

(iii) Addition of dC tail

This double-stranded DNA was subjected to the reaction of 60 units of nuclease S1 (Bethesda Research Laboratories) in 50 µl of a reaction mixture (0.1 M sodium acetate, pH 4.5, 0.25 M NaCl, 1.5 mM ZnSO₄) at room temperature for 30 minutes. The reaction mixture was then deproteinized with phenol, and the DNA was precipitated with ethanol. The DNA was subjected to the reaction of 30 units of terminal transferase (Bethesda Research Laboratories) in a reaction mixture [0.14 M potassium cacodylate, 0.3 M Tris (base), pH 7.6, 2 mM dithiothreitol, 1 mM CoCl₂, 0.15 mM dCTP] at 37°C for 3 minutes, whereby about 20 deoxycytidylates were linked to each 3'-end of the double-stranded DNA. The above series of reactions gave about 300 ng of a deoxycytidylate chain-bearing double-stranded DNA.

(iv) Cleavage of Escherichia coli plasmid and addition of dG tail

Separately, 10 µg of Escherichia coli plasmid pBR322 DNA was subjected to the reaction of 20 units of the restriction enzyme PstI in 50 µl of a reaction mixture [50 mM NaCl, 6 mM Tris·HCl (pH 7.4), 6 mM MgCl₂, 6 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin] at 37°C for 3 hours, whereby the pBR322 DNA was cleaved at the PstI recognition site. After deproteinization with phenol, the cleavage product was further subjected to the reaction

of 30 units of terminal transferase in 50 μ l of a reaction mixture [0.14 M potassium cacodylate, 0.3 M Tris (base), pH 7.6, 2 mM dithiothreitol, 1 mM CoCl_2 , 0.15 mM dGTP] at 37°C for 3 minutes, whereby the above plasmid pBR322 DNA was extended by about 8 deoxyguanylates at each 3'-end.

(v) Annealing of cDNA and Escherichia coli plasmid and transformation of Escherichia coli

The thus-obtained synthetic double-stranded DNA (0.1 μ g) and the above plasmid pBR322 (0.5 μ g) were annealed together by heating in a solution comprising 0.1 M NaCl, 50 mM Tris-HCl, pH 7.6, and 1 mM EDTA at 65°C for 2 minutes and then at 45°C for 2 hours, followed by slow cooling. The transformation of Escherichia coli χ 1776 was performed according to the method of Enea et al. [J. Mol. Biol., 96, 495 (1975)].

(vi) Isolation of cDNA-containing plasmid

In this way, 1445 tetracycline -resistant colonies were isolated. The DNA of each of them was fixed on a nitrocellulose filter (vide supra).

Separately, the gene fragment corresponding to the human IgE H-chain polypeptide (vide supra) was labelled with ^{32}P by the nick translation method (vide supra).

The DNA (0.2 μ g) was treated in 25 μ l of a reaction mixture [50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2 , 1 mM β -mercaptoethanol, 10 μ Ci α - ^{32}P -dATP, 0.4 ng bovine pancreatic DNase I (Werthington)] at room temperature for 2 minutes. Then, 25 units of Escherichia coli DNA polymerase I (Boehringer Mannheim) was added and the reaction was conducted at 15°C for 30 minutes. Purification by extraction with phenol and precipitation with ethanol gave a uniformly ^{32}P -labelled DNA.

With this ^{32}P -DNA as the probe, this was annealed with the DNA fixed on the nitrocellulose filter according to the method of Lawn et al. [Nucleic Acids Res., 9, 6103 (1981)]. As a result of autoradiography, 9 colonies responding to the probe were isolated and named pGET 1 to 9, respectively.

The plasmid DNA was isolated from cells of each of these colonies by the method of Birnboim-Doly [Birnboim, H. C. and Doly, J., Nucleic Acids Res. 7, 1513 (1979)].

Then, the insert was cut out from the plasmid DNA using the restriction enzyme PstI, whereby, among the plasmids separated, pGET2 DNA was found to contain the longest insert. Accordingly, the pGET2 DNA was selected for further use.

The restriction enzyme cleavage map of the cDNA inserted in this plasmid is as shown in Fig. 3. The primary structure (nucleotide sequence) of the cDNA sequence as inserted in the pGET2 plasmid was determined by the dideoxynucleotide synthetic chain termination method and by the method of Maxam-Gilbert. The nucleotide sequence thus determined is as shown in Fig. 4. The polynucleotide of the nucleotide sequence 18-1502 as shown in Fig. 4 corresponds to the polynucleotide as shown in Fig. 1.

The amino acid sequence which this nucleotide sequence codes for, when there is no reading frame shift, is approximately equal to the amino acid sequence of the IgE H-chain polypeptide as reported by Dorrington et al. [Immunological Rev., 41, 3 (1978)]. This confirms that the cDNA inserted in pGET2 codes for the IgE H-chain polypeptide. This cDNA begins with the codon coding for the 63th amino acid in the V region of the IgE H-chain as reported by Dorrington et al. (vide supra), hence wholly codes for the C region. Furthermore, it is believed that it retains the whole structure on the 3'-end side of the mRNA, inclusive of non-coding regions, since the poly(A) structure is present.

Therefore, the C-region polypeptide which carries the antigenicity of human IgE can be produced by adding the translation start codon ATG to the 5'-end of the nucleotide sequence inserted in the above plasmid, without reading frame shift, followed by insertion into another expression plasmid and transformation of Escherichia coli, for instance, therewith.

Example 2

The insert in the plasmid pGET2 as obtained in Example 1 was cut out using the restriction enzyme PstI. This DNA fragment (2 µg) was partially digested from both ends under the reaction of 2 units of nuclease Bal 31 [New England Biolabs; Gray et al., Nucleic Acids Res., 2, 1459 (1975)] in 60 µl of a reaction mixture (20 mM Tris·HCl, pH 8.0, 0.6 M NaCl, 12 mM CaCl₂, 12 mM MgCl₂, 1 mM EDTA) at 30°C for 1 minute.

The DNA was extracted from the reaction mixture with phenol and purified by precipitation with ethanol, and then joined with the adapter 5'CATCGATG^{3'}, which contains the translation start codon and restriction enzyme ClaI-recognition site, using T4 DNA ligase (New England Biolabs).

Separately, the plasmid ptrp771 as an expression plasmid (the vector being pBR322), which contains an Escherichia coli trp promoter portion [promoter- and operator-containing 276 bp DNA fragment; Bennett, G. N. et al., J. Mol. Biol., 121, 113 (1978)], was constructed according to the method disclosed in Japanese Patent Application No. 57-85280/1982.

This expression plasmid ptrp771 was cleaved with the restriction enzyme ClaI. Thereinto, at the cleavage site, inserted was the above-mentioned pGET2 insert DNA-adapter joining product, which also had been cleaved with ClaI, with the use of T4 DNA ligase (Fig. 5). Using the reaction product, Escherichia coli 294 was transformed according to the method of Cohen et al. (vide supra).

There were obtained a large number of colonies containing plasmids differing in the nuclease Bal 31 digestion region.

Human IgE H-chain polypeptide-producing colonies were selected from among the thus-obtained colonies by the colony immunoassay method [Kemp, D. J. and Cowman, A. F., Proc. Natl. Acad. Sci. USA, 78, 4520 (1981)]. Thus, the colonies grown on a nitrocellulose filter were lysed by contacting with a 0.1 M NaHCO₃-0.1% Triton X100-lysozyme (200 µg/ml) solution and directly transferred onto a cyanogen bromide-activated filter paper (Whatman No. 540) for fixation of the colonies on the filter paper. The filter paper was reacted with goat antihuman IgE antibody (Miles), then washed with a washing solution (50 mM Tris·HCl, pH 8.0, 0.5 M NaCl, 0.1% Triton X100, 1% bovine serum albumin), and further reacted with ¹²⁵I-labelled protein A (RCC Amersham, Great Britain). After the reaction, the filter paper was washed well and autoradiographed.

The plasmid contained in the colony that reacted most positively with the anti-human IgE antibody in the above assay was named pGETtrp104. This plasmid was extracted from cells by the method of Birnboim-Doly (vide supra). The nucleotide sequence coding for the human IgE H-chain, which was inserted in ptrp771 and now existing in this pGETtrp104, was determined by the dideoxynucleotide synthetic chain termination method (vide supra). It was revealed that the human IgE H-chain polypeptide-encoding polynucleotide starting with the codon for the 92nd amino acid (according to the report by Dorrington) is located

following the translation start codon without reading frame shift and that the poly(A) structure at the end of the mRNA structure is retained on the 3'-end side (Fig. 4). Escherichia coli 294/pGETtrp104 has been deposited with the Institute for Fermentation, Osaka under deposit No. IFO 14284.

Example 3

(i) From the plasmid PGET2 as obtained in Example 1, the insert was cut out with the restriction enzyme PstI. This DNA fragment was further cleaved with the restriction enzyme SalI. There was thus obtained an about 1,150 bp DNA fragment having the SalI site at one end and the PstI site at the other. The single-stranded cohesive DNA terminus at the SalI site of this DNA fragment was filled in with Escherichia coli DNA polymerase I large fragment and the DNA fragment was joined with the adapter 5'GCATCGATGC^{3'} containing the translation start codon and restriction enzyme ClaI recognition site with the use of T4 DNA ligase (New England Biolabs). The joining product was cleaved with the restriction enzyme ClaI and then joined with the expression plasmid ptrp771 cleaved in advance with the restriction enzymes ClaI and PstI, with the use of T4 DNA ligase (Fig. 6). The above series of reactions resulted in construction of a human IgE H-chain polypeptide expression plasmid, pGETtrp302, containing the translation start codon and the Leu-encoding codon CTC newly introduced without reading frame shift at a site downstream from the trp promoter, and coding for the human IgE H-chain polypeptide starting from the codon for the 218th amino acid according to the report of Dorrington. Escherichia coli 294 was transformed with this expression plasmid according to the method of Cohen et al. to give a desired strain carrying the plasmid pGETtrp302.

(ii) From the plasmid pGET2 as obtained in Example 1, the insert was cut out with the restriction enzyme PstI. This DNA fragment was further cleaved with the restriction

enzyme HinfI. There was thus obtained an about 810 bp DNA fragment with the HinfI site at one end and the PstI site at the other.

The single-stranded cohesive DNA terminus at the HinfI site of this DNA fragment was filled in with Escherichia coli DNA polymerase I large fragment (Bethesda Research Laboratories) so as to render the end blunt, and then the DNA fragment was joined with the same adapter as used in Example 3-(i), i.e. 5'GCATCGATGC^{3'}, with the use of T4 DNA ligase.

The joining product was cleaved with the restriction enzyme ClaI and joined with the expression plasmid ptrp771 cleaved in advance with the restriction enzymes ClaI and PstI, with the use of T4 DNA ligase (Fig. 6). The above series of reactions resulted in construction of a human IgE H-chain polypeptide expression plasmid, pGETtrp410, containing the translation start codon and the codon CAT for His at a site downstream from the trp promoter, and coding for the human IgE H-chain polypeptide starting with the codon for the 331st amino acid according to Dorrington without reading frame shift. Escherichia coli 294 was transformed with this plasmid according to the method of Cohen et al. to give a desired strain carrying the plasmid pGETtrp410.

Example 4

The IgE H-chain expression plasmid-carrying strains as obtained in Examples 2 and 3 were cultivated in 20 ml of M9 medium containing 1% glucose and 0.4% Casamino acids at 37°C for 4 hours. Then, indolyl acrylic acid was added to a concentration of 30 µg/ml, and the cultivation was continued at 37°C for 3 hours. Cells were harvested, washed with saline, and lysed by suspending in 0.5 ml lysing solution (10 mM Tris·HCl, pH 8.0, 10 mM EDTA,

0.2 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.02% Triton X100, 0.1 mg/ml lysozyme) and allowing the suspension to stand at 0°C for 45 minutes and then at 37°C for 2 minutes. The lysate was further subjected to slight (30-second) untrasonic treatment for breaking cellular DNAs which were dissolved. The lysate was then centrifuged at 4°C at 15,000 rpm (Sorvall SS34 rotor) for 30 minutes. The thus-obtained supernatant was assayed for IgE activity by the RIST method (vide supra) using an IgE assay kit (IgE Test-Shionogi; Shionogi).

The results are shown in Table 2. The strain carrying pGETtrp302 produced the human IgE H-chain polypeptide at the highest rate (480 ng/ml extract).

Table 2

Transformant	IgE H-chain production (ng/ml extract)
<u>E. coli</u> 294 (ptrp771)	0
<u>E. coli</u> 294 (pGETtrp104)	84
<u>E. coli</u> 294 (pGETtrp302)	480
<u>E. coli</u> 294 (pGETtrp410)	48

Example 5

Anti-human IgE monoclonal antibody was bound to a water-insoluble carrier Affigel 10 (Bio-Rad) by the method described in Reference Example 2 of Japanese Patent Application No. 19,324/1981.

5 ml of the extract from pGETtrp302-carrying cells as obtained in Example 4 was treated on a 1-ml column of the anti-human IgE monoclonal antibody-Affigel 10. The column was washed with 50 ml of PBS (20 mM phosphate buffer, pH 6.8, 0.15 M NaCl) containing 20% dextrose, and the human IgE H-chain adsorbed on the column was eluted from the column with 5 ml of 0.2 M acetic acid-0.15 M NaCl solution. The eluate was immediately neutralized, and dialyzed against 1 liter of PBS at 5°C for 24 hours. This procedure gave the human IgE H-chain polypeptide in a purity of not lower than 80% at a recovery rate of about 50%.

4. Brief Description of the Drawings

Fig. 1 illustrates the nucleotide sequence coding for the human IgE H-chain polypeptide, Fig. 2 illustrates the amino acid sequence corresponding to the nucleotide sequence shown in Fig. 1, Fig. 3 shows the restriction enzyme map for the cDNA in pGET2 as obtained in Example 1, and Fig. 4 illustrates the primary structure (nucleotide sequence) of said cDNA. Fig. 5 shows the construction scheme in Example 2, and Fig. 6 shows the construction scheme in Example 3. The portion indicated by //// represents the human IgE H chain-encoding fragment.

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Attorney: Sakuji Amai

Figure 1

	10	20	30	40	50	
	* *		* *		* *	
	AGATTT	CAGGG	CAGGG	TCACCAT	GACCAGAG	ACGGTCCTTCAGTACAGC
	TCTAAAG	TCCCG	TCCCG	AGTGGTACT	GGTCTCTGCGC	AGGAAGTCATGTCG
						50
	* *		* *		* *	
	CTACAT	GGACCT	GAGAAGT	CTGAGAT	CTGACGACT	CGGCCGTGTTTTACT
	GATGTAC	CTGGACT	CTTCAGACT	CTAGACT	GCTGAGCCGGC	ACAAAATGA
						100
	* *		* *		* *	
	GTGCG	AAAAGT	GACCC	TTTTT	GGAGT	GATTATTATAACTTTGACTACTCG
	CACGCT	TTTTT	CACTG	GGGAAAA	ACCTCACTA	AATAATTGAAACTGATGAGC
						150
	* *		* *		* *	
	TACACT	TTGGAC	GCTCTG	GGGGCC	AAGGG	ACCACGGTCACCGTCTCCTCAGC
	ATGTGA	AACCTG	CAGAC	CCCGG	TTCCCTG	GTCAGTGCCAGAGGAGTCG
						200
	* *		* *		* *	
	CTCCAC	ACAGAG	CCCATC	CGTCTT	CCCCCT	TGACCCGCTGCTGCAAAAACA
	GAGGTG	TGTCTC	GGGTAG	GCAGAA	GGGGA	ACTGGGCGACGACGTTTTTGT
						250
	* *		* *		* *	
	TTCCCT	CCAATG	CCACCT	CCGTG	ACTCTG	GGGCTGCCTGGCCACGGGCTAC
	AAGGG	AGGTTA	CGGTG	GAGGCA	CTGAGAC	CCCGACGGACCGGTGCCCGATG
						300
	* *		* *		* *	
	TTCCCG	GAGCCG	GTGATG	GTGAC	CTGGG	ACACAGGCTCCCTCAACGGGAC
	AAGGG	CCTCGG	CCACTA	CCACTG	GACCTG	TGTGTCGAGGGAGTTGCCCTG
						350
	* *		* *		* *	
	AACTAT	GACCTT	ACCAGC	CACCA	CCCTCA	CGCTCTCTGGTCACTATGCCA
	TTGATA	CTGGA	ATGGT	CGGTG	GGTGG	GAGTGCGAGAGACCAGTGATACGGT
						400
	* *		* *		* *	
	CCATCA	GCTTG	CTGAC	CGTCT	CGGGT	GCGTGGGCCAAGCAGATGTTACCC
	GGTAGT	CGAAC	GACTGG	CAGAG	CCCA	CGCACCCCGTTCTGTACAAGTGG
						450
	* *		* *		* *	
	TGCCGT	GTTGG	CACACA	CTCCAT	CGTCC	ACAGACTGGGTGCGACAACAAAC
	ACGGC	ACAC	CCGTG	TGTG	AGGTAG	CAGGTGTCTGACCCAGCTGTTGTTTTG
						500

..continued

Figure 1

	10	20	30	40	50	
	*	*	*	*	*	
CTTCAGCGTCTGCTCCAGGGACTTCACCCCGCCACCGTGAAGATCTTAC						550
GAAGTCGCAGACGAGGTCCCTGAAGTGGGGCGGGTGGCACTTCTAGAATG						
	*	*	*	*	*	
AGTCGTCTGCGACGGCGGGCGGGCACTTCCCCCGACCATCCAGCTCCTG						600
TCAGCAGGACGCTGCCGCCGCCGTGAAGGGGGCTGGTAGGTGAGGAC						
	*	*	*	*	*	
TGCCTCGTCTCTGGGTACACCCAGGGACTATCAACATCACCTGGCTGGA						650
ACGGAGCAGAGACCCATGTGGGGTCCCTGATAGTTGTAGTGGACCGACCT						
	*	*	*	*	*	
GGACGGGCAGGTTCATGGACGTGGACTTGTCCACCGCCTCTACCACGCAGG						700
CCTGCCCGTCCAGTACCTGCACCTGAACAGGTGGCGGAGATGGTGGCTCC						
	*	*	*	*	*	
AGGGTGAGCTGGCCTCCACACAAAGCGAGCTCACCTCAGCCAGAAAGCAC						750
TCCCACTCGACCGGAGGTGTGTTTCGCTCGAGTGGGAGTCGGTCTTCGTG						
	*	*	*	*	*	
TGGCTGTCAGACCGCACCTACACCTGCCAGGTACCTATCAAGGTCACAC						800
ACCGACAGTCTGGCGTGGATGTGGACGGTCCAGTGGATAGTTCCAGTGTG						
	*	*	*	*	*	
CTTTGAGGACAGCACCAAGAAGTGTGCAGATTCCAACCCGAGAGGGGTGA						850
GAAACTCCTGTCGTGGTTCTTCACACGTCTAAGGTTGGGCTCTCCCCACT						
	*	*	*	*	*	
GCGCCTACCTAAGCCGGCCCGCCGTTCCGACCTGTTTCATCCGCAAGTCG						900
CGCGGATGGATTCCGGCCGGGTCCGGCAAGCTGGACAAGTAGGCGTTACAGC						
	*	*	*	*	*	
CCCACGATCACCTGTCTGGTGGTGGACCTGGCACCAGCAAGGGGACCGT						950
GGGTGCTAGTGGACAGACCACCACCTGGACCGTGGGTGCTTCCCTGGCA						
	*	*	*	*	*	
GAACCTGACCTGGTCCCGGGCCAGTGGGAAGCCTGTGAACCACTCCACCA						1000
CTTGGACTGGACAGGGCCCGGTACCCCTTCCGACACTTGGTGAGGTGGT						

.. coninued

Figure 1

10	20	30	40	50	
* * * * *					
GAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCACGTCCACCCCTG					1050
CTTTCCTCCTCTTCGTGCGGTTACCGTGCAATTGGCAGTGCAGGTGGGAC					
* * * * *					
CCGGTGGGCACCCGAGACTGGATCGAGGGGGAGACCTACCAGTGCAGGGT					1100
GGCCACCCGTGGGCTCTGACCTAGCTCCCCCTCTGGATGGTCACGTCCCA					
* * * * *					
GACCCACCCCCACCTGCCAGGGGCCCTCATGCGGTCCACGACCAAGACCA					1150
CTGGGTGGGGGTGGACGGGTCCCGGGAGTACGCCAGGTGCTGGTTCTGGT					
* * * * *					
GCGGCCCGCGTGCTGCCCGGAAGTCTATGCGTTTGCGACGCCGGAGTGG					1200
CGCCGGGCGCACGACGGGGCCTTCAGATACGCAAACGCTGCGGCCCTCACC					
* * * * *					
CCGGGGAGCCGGGACAAGCGCACCCCTCGCCTGCCTGATCCAGAACTTCAT					1250
GGCCCCCTCGGCCCTGTTGCGGTGGGAGCGGACGGACTAGGTCTTGAAGTA					
* * * * *					
GCCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTGCAGCTCCCGG					1300
CGGACTCCTGTAGAGCCACGTACCGACGTGTTGCTCCACGTGAGGGCC					
* * * * *					
ACGCCCGGCACAGCACGACGACGCCCGCAAGACCAAGGGCTCCGGCTTC					1350
TGCGGGCCGTGTCGTGCTGCGTGGGGCGTTCTGGTTCCCGAGGCCGAAG					
* * * * *					
TTCGTCTTCAGCCGCCTGGAGGTGACCAGGGCCGAATGGGAGCAGAAAGA					1400
AAGCAGAAGTCGGCGGACCTCCACTGGTCCCGGCTTACCCTCGTCTTTCT					
* * * * *					
TGAGTTCATCTGCCGTGCAGTCCATGAGGCAGCGAGCCCTCACAGACCG					1450
ACTCAAGTAGACGGCACGTACGGTACTCCGTGCTCGGGGAGTGTCTGGC					
* * * * *					
TCCAGCGAGCGGTGTCTGTAAATCCCGGTAAATGA					
AGGTGCGCTCGCCACAGACATTTAGGGCCATTTACT					

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Figure 2

ARG PHE GLN GLY ARG VAL THR MET THR ARG ASP ALA SER PHE SER
THR ALA TYR MET ASP LEU ARG SER LEU ARG SER ASP ASP SER ALA
VAL PHE TYR CYS ALA LYS SER ASP PRO PHE TRP SER ASP TYR TYR
ASN PHE ASP TYR SER TYR THR LEU ASP VAL TRP GLY GLN GLY THR
THR VAL THR VAL SER SER ALA SER THR GLN SER PRO SER VAL PHE
PRO LEU THR ARG CYS CYS LYS ASN ILE PRO SER ASN ALA THR SER
VAL THR LEU GLY CYS LEU ALA THR GLY TYR PHE PRO GLU PRO VAL
MET VAL THR TRP ASP THR GLY SER LEU ASN GLY THR THR MET THR
LEU PRO ALA THR THR LEU THR LEU SER GLY HIS TYR ALA THR ILE
SER LEU LEU THR VAL SER GLY ALA TRP ALA LYS GLN MET PHE THR
CYS ARG VAL ALA HIS THR PRO SER SER THR ASP TRP VAL ASP ASN
LYS THR PHE SER VAL CYS SER ARG ASP PHE THR PRO PRO THR VAL
LYS ILE LEU GLN SER SER CYS ASP GLY GLY GLY HIS PHE PRO PRO
THR ILE GLN LEU LEU CYS LEU VAL SER GLY TYR THR PRO GLY THR
ILE ASN ILE THR TRP LEU GLU ASP GLY GLN VAL MET ASP VAL ASP
LEU SER THR ALA SER THR THR GLN GLU GLY GLU LEU ALA SER THR
GLN SER GLU LEU THR LEU SER GLN LYS HIS TRP LEU SER ASP ARG
THR TYR THR CYS GLN VAL THR TYR GLN GLY HIS THR PHE GLU ASP
SER THR LYS LYS CYS ALA ASP SER ASN PRO ARG GLY VAL SER ALA
TYR LEU SER ARG PRO SER PRO PHE ASP LEU PHE ILE ARG LYS SER
PRO THR ILE THR CYS LEU VAL VAL ASP LEU ALA PRO SER LYS GLY

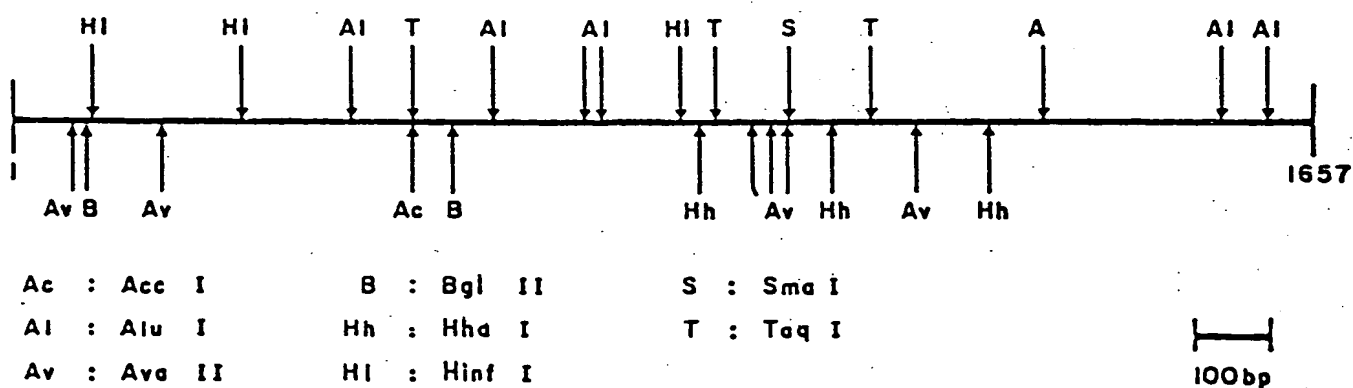
...continued

Figure 2

THR VAL ASN LEU THR TRP SER ARG ALA SER GLY LYS PRO VAL ASN
HIS SER THR ARG LYS GLU GLU LYS GLN ARG ASN GLY THR LEU THR
VAL THR SER THR LEU PRO VAL GLY THR ARG ASP TRP ILE GLU GLY
GLU THR TYR GLN CYS ARG VAL THR HIS PRO HIS LEU PRO ARG ALA
LEU MET ARG SER THR THR LYS THR SER GLY PRO ARG ALA ALA PRO
GLU VAL TYR ALA PHE ALA THR PRO GLU TRP PRO GLY SER ARG ASP
LYS ARG THR LEU ALA CYS LEU ILE GLN ASN PHE MET PRO GLU ASP
ILE SER VAL GLN TRP LEU HIS ASN GLU VAL GLN LEU PRO ASP ALA
ARG HIS SER THR THR GLN PRO ARG LYS THR LYS GLY SER GLY PHE
PHE VAL PHE SER ARG LEU GLU VAL THR ARG ALA GLU TRP GLU GLN
LYS ASP GLU PHE ILE CYS ARG ALA VAL HIS GLU ALA ALA SER PRO
SER GLN THR VAL GLN ARG ALA VAL SER VAL ASN PRO GLY LYS - -

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Attorney: Sakuji Amai

Figure 3



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 Attorney: Sakuji Amai

Figure 4

10	20	30	40	50	
* * * * *					
GGGGGGGGGGGGGGGGGAGATTTTCAGGGCAGGGTCACCATGACDAGAGAC					50
CCCCCCCCCCCCCGCTCTAAAGTCCCGTCCCAGTGGTACTGGTCTCTG					
* * * * *					
GCGTCCTTCAGTACAGCCTACATGGACCTGAGAAGTCTGAGATCTGACGA					100
CGCAGGAAGTCATGTCCGATGTACCTGGACTCTTCAGACTCTAGACTGCT					
* * * * *					
CTCGGCCGTGTTTTACTGTGCGAAAAGTGACCCTTTTTTGGAGTGATTATT					150
GAGCCGGCACAAAATGACACGCTTTTCACTGGGAAAACCTCACTAATAA					
* * * * *					
ATAACTTTGACTACTCGTACACTTTTGGACGTCTGGGGCCAGGGACDAGG					200
TATTGAAACTGATGAGCATGTGAAACCTGCAGACCCCGGTTCCCTGGTGCC					
* * * * *					
GTCACCGTCTCCTCAGCCTCCACACAGAGCCCATCCGTCTTCCCCTTGAC					250
CAGTGGCAGAGGAGTCGGAGGTGTGTCTCGGGTAGGCAGAAAGGGGAAGTG					
* * * * *					
CCGCTGCTGCAAAAACATTCCCTCCAATGCCACCTCCGTGACTCTGGGGCT					300
GGCGACGACGTTTTTTGTAAGGGAGGTTACGGTGGAGGCACTGAGACCCGA					
* * * * *					
GCCTGGCCACGGGGCTACTTCCCGGAGCCGGTGATGGTGACCTGGGACACA					350
CGGACCGGTGCCCCGATGAAGGGCCCTCGGCCACTACCACTGGACCCTGTGT					
* * * * *					
GGCTCCCTCAACGGGACAACTATGACCTTACCAGCCACCACCCTCAGGCT					400
CCGAGGGAGTTGCCCTGTTGATACTGGAATGGTCGGTGGTGGGAGTGCGA					
* * * * *					
CTCTGGTCACTATGCCACCATCAGCTTGCTGACCGTCTCGGGTGGCTGGG					450
GAGACCAGTGATACGGTGGTAGTGCAGACGACTGGCAGAGCCCACGCACCC					
* * * * *					
CCAAGCAGATGTTACCTGCCGTGTGGCACAACACTCCATCGTCCACAGAC					500
GGTTCGTCTACAAGTGGACGGCACACCCTGTGTGAGGTAGCAGGTGTCTG					

..continued

Figure 4

	10	20	30	40	50	
	*	*	*	*	*	
TGGGTCGACAACAAACCTTCAGCGTCTGCTCCAGGGACTTCACCCCGCC						550
ACCCAGCTGTTGTTTTGGAAGTCGCAGACGAGGTCCCTGAAGTGGGGCGG						
	*	*	*	*	*	
CACCGTGAAGATCTTACAGTCGTCTCGGACGGCGGGCGGGCACTTCCCCC						600
GTGGCACTTCTAGAATGTCAGCAGGACGCTGCCGCCGCCCGTGAAGGGGG						
	*	*	*	*	*	
CGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCCAAGGGACTATC						650
GCTGGTAGGTGAGGACACGGAGCAGAGACCCATGTGGGGTCCCTGATAG						
	*	*	*	*	*	
AACATCACCTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCAC						700
TTGTAGTGGACCGACCTCCTGCCCGTCCAGTACCTGCACCTGAACAGGTG						
	*	*	*	*	*	
CGCCTCTACCACGCAGGAGGGTGAGCTGGCCTCCACACAAAGCGAGCTCA						750
GCGGAGATGGTGCGTCCTCCCACTCGACCGGAGGTGTGTTTCGCTCGAGT						
	*	*	*	*	*	
CCCTCAGCCAGAAGCACTGGCTGTCAGACCGCACCTACACCTGCCAGGTC						800
GGGAGTCGGTCTTCGTGACCGACAGTCTGGCGTGGATGTGGACGGTCCAG						
	*	*	*	*	*	
ACCTATCAAGGTCACACCTTTGAGGACAGCACCAAGAAGTGTGCAGATTC						850
TGGATAGTTCCAGTGTGGAAACTCCTGTCTGTTCTTCACACGTCTAAG						
	*	*	*	*	*	
CAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGGCCAGCCCGTTCCGACC						900
GTTGGGCTCTCCCCACTCGCGGATGGATTCCGGCCGGGTCCGGGCAAGCTGG						
	*	*	*	*	*	
TGTTTCATCCGCAAGTCGCCACGATCACCTGTCTGGTGGTGGACCTGGCA						950
ACAAGTAGGCGTTTCAGCGGGTGCTAGTGGACAGACCACCACCTGGACCGT						
	*	*	*	*	*	
CCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGGCCAGTGGGAAGCC						1000
GGGTGCTTCCCTGGCACTTGGACTGGACAGGGCCCGGTACCCCTTCGG						

..continued

Figure 4

10	20	30	40	50	
* * * *					
TGTGAACCACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCAGCTTAA					1050
ACACTTGGTGAGGTGGTCTTTCTCTCTTTCGTGCGCTTACCGTGCAATT					
* * * *					
CCGTCAAGTCCACCCTGCCGGTGGGCACCCGAGACTGGATCGAGGGGGAG					1100
GGCAGTGCAGGTGGGACGGCCACCCGTGGGCTCTGACCTAGCTCCCCCTC					
* * * *					
ACCTACCAGTGCAGGGTGACCCACCCCACTGCCAGGGCCCTCATGCG					1150
TGGATGGTCAAGTCCCACTGGGTGGGGGTGGACGGGTCCCGGGAGTACGC					
* * * *					
GTCCACGACCAAGACCAGCGGCCCGCGTGTCTGCCCGGAAGTCTATGCGT					1200
CAGGTGCTGGTTCTGGTGC CGGGCGCAGCAGGGGCTTCAGATACGCA					
* * * *					
TTGCGACGCCGGAGTGGCCGGGGAGCCGGGACAAGCGCACCCCTCGCCTGC					1250
AACGCTGCGGCCTCACCGGCCCTCGGCCCTGTTGCGGTGGGAGCGGACG					
* * * *					
CTGATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCACAA					1300
GACTAGGTCTTGAAGTACGGACTCCTGTAGAGCCACGTCACCGACGTGTT					
* * * *					
CGAGGTGCAGCTCCCGGACGCCCGGCACAGCAGCAGCGAGCCCGCAAGA					1350
GCTCCACGTGCAGGGCCTGCGGGCCGTGTCTGTGCTGCGTGGGGGCGTTCT					
* * * *					
CCAAGGGCTCCGGCTTCTTCGTCTTCAGCCGCCTGGAGGTGACCAGGGCC					1400
GGTTCCCGAGGCCGAAGAAGCAGAAGTCGGCGGACCTCCACTGGTCCCGG					
* * * *					
GAATGGGAGCAGAAAGATGAGTTTCATCTGCCGTGCAGTCCATGAGGCAGC					1450
CTTACCCTCGTCTTTCTACTCAAGTAGACGGCAGCTCAGGTAATCCGTCG					
* * * *					
GAGCCCCCTCACAGACCGTCCAGCGAGCGGTGTCTGTAAATCCCGGTAAAT					1500
CTCGGGGAGTGTCTGGCAGGTGCTCGCCACAGACATTTAGGGCCATTTA					

..continued

Figure 4

10	20	30	40	50	
	*	*	*	*	*
GACGTACTCCTGCCTCCCTCCCTCCCAGGGCTCCATCCAGCTGTGCAGTG					1550
CTGCATGAGGACGGAGGGAGGGAGGGTCCCGAGGTAGGTCGACACGTCAC					
	*	*	*	*	*
GGGAGGACTGGCCAGACCTTCTGTCCACTGTTGCAATGACCCAGGAAGC					1600
CCCTCCTGACCGGTCTGGAAGACAGGTGACAACGTTACTGGGGTCCTTCG					
	*	*	*	*	*
TACCCCAATAAACTGTGCCTGCTCAGAAAAAAAAAAAAAAAAACCCCCC					1650
ATGGGGGTTATTTGACACGGACGAGTCTTTTTTTTTTTTTTTTTGGGGGGG					
	*	*	*	*	*
CCCCCCC					
GGGGGGG					

TOTAL NUMBER OF NUCLEOTIDE PAIRS = 1657

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Attorney: Sakuji Amai

Figure 5

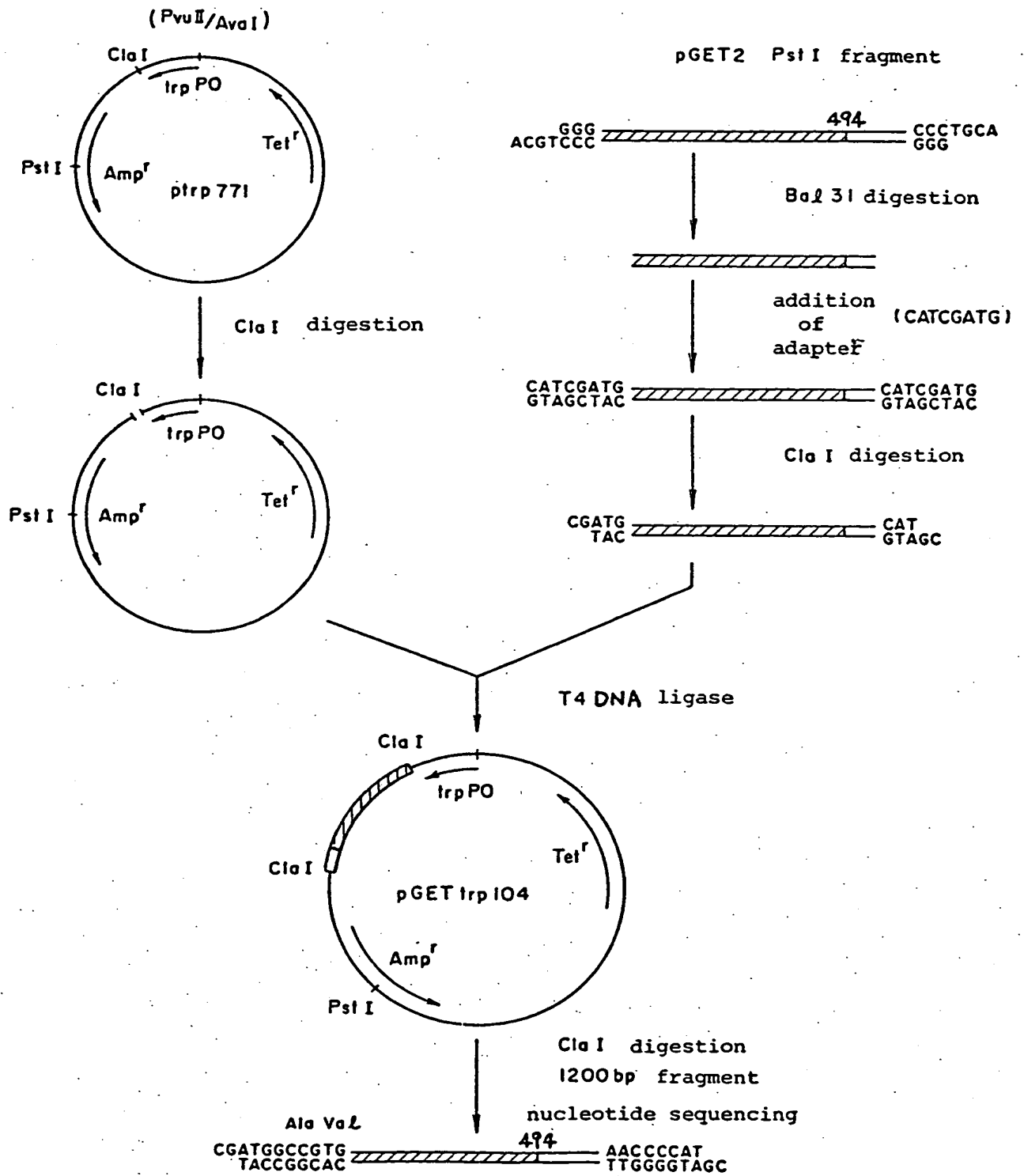
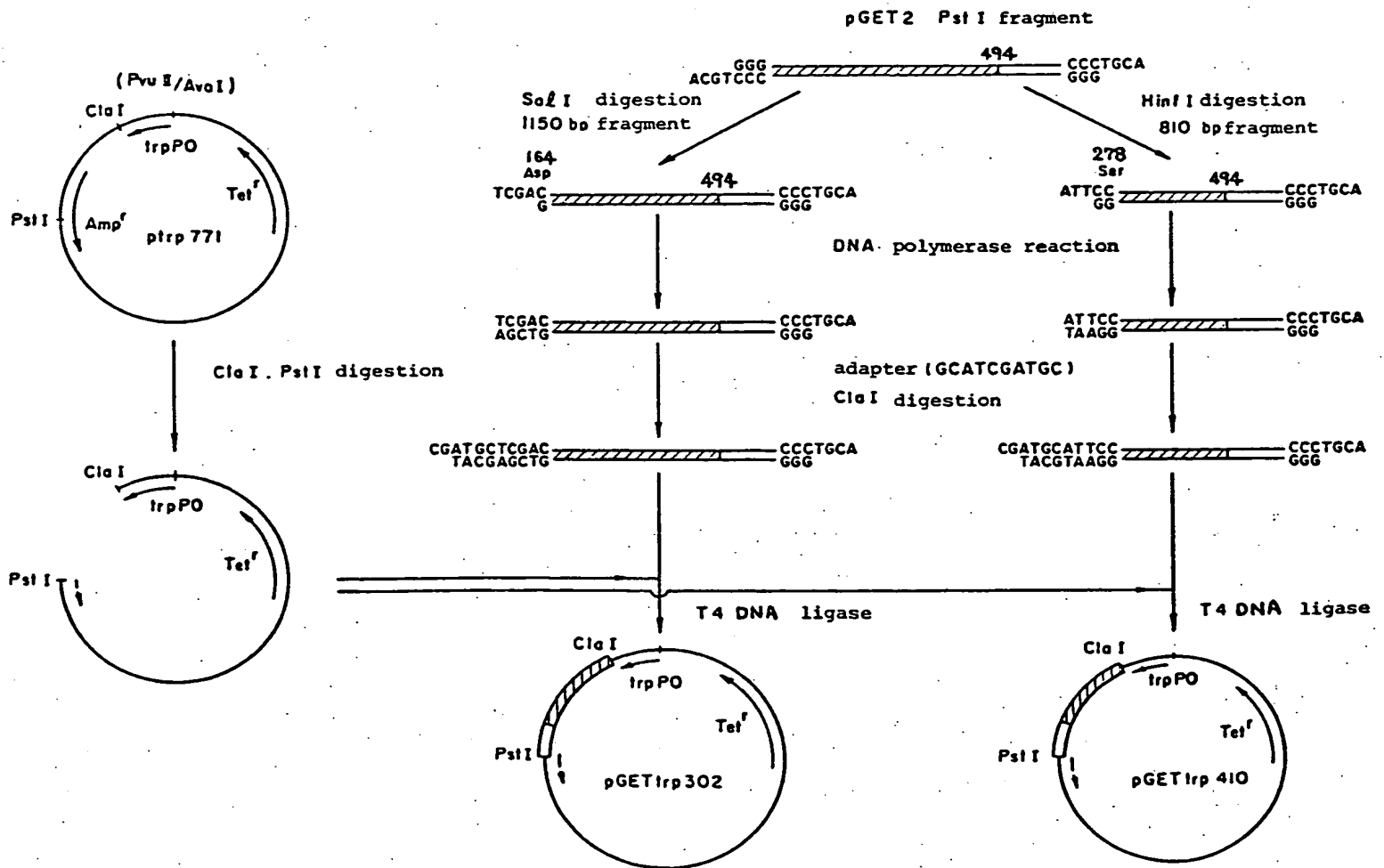


Figure 6



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